

# Biopharmaceutical Powders: Particle Formation and Formulation Considerations

Yuh-Fun Maa\* and Steven J. Prestrelski

*PowderJect Technologies, 6511 Dumbarton Circle, Fremont, CA 94555, U.S.A.*

**Abstract:** It is well known that protein/peptide-based drug formulations are more stable in the solid state than in the liquid state, thereby offering stability advantages in ambient temperature storage, product shipping/distribution, and long-term shelf life. Novel powder-based drug delivery systems recently emerging for applications in sustained release, inhalation, intradermal delivery, etc. add more value to protein solid dosage forms. Despite great research interests in understanding the drying effects on protein stability and a large collection of publications focusing on this area, systematic accounts of powder formation techniques are lacking. This review is to summarize a number of methods currently available for protein powder preparation. Some are common methods such as lyophilization, spray drying, pulverization, and precipitation, and some methods are more recently developed such as supercritical fluid precipitation, spray-freeze drying, fluidized-bed spray coating and emulsion precipitation. In addition to examining the individual process effect on protein stability that is always the focus of formulation scientists, this review also likes to evaluate each method from a more practical sense in terms of process versatility and scalability. The conclusion is that each method has its own advantages and the use of a method is formulation and application specific. With the understanding of the principles and advantages of these methods, it can benefit our choice on selecting appropriate techniques for preparing a desired protein powder formulation for specific applications.



## I. BACKGROUND

Pharmaceutical powder production and handling have long been an integral part of oral dosage form preparation. Some particle-formation methods such as precipitation, granulation, pulverization, and spray drying are therefore well established [1]. However, when it comes to biotechnology, oral delivery of protein products found little luck because of the harsh environment in the gastrointestinal route. Furthermore, all of the approved protein products are delivered through parenteral, primarily the subcutaneous or the intramuscular, routes. With the concept that proteins/peptides are more stable in the solid state, many protein products were prepared into solid dosage forms, mainly by freeze drying. These products, however, are still delivered parenterally after being reconstituted. For a long time, formulation of stable freeze-dried proteins has been the whole-hearted goal of most protein formulation scientists. Over the last decade, the search for alternative protein delivery

routes/methods has attracted a great deal of scientific interest. Of these methods, some are powder based including microspheres/microcapsules for long-acting delivery, fine powders for pulmonary delivery, and biopharmaceutical as well as vaccine powders for intradermal delivery. Therefore, the efforts to identify appropriate powder formation methods are mounting. Still, the suitability of specific powder formation methods as valued by its effect on protein stability remains the focus of formulation scientists. However, process issues are also fundamentally important in development of pharmaceutical products. Studies on process optimization are quite limited. Therefore, we like to review the currently available powder formation processes with the focus on novel methods emerging recently such as spray-freeze drying [2-3], supercritical fluid methods [4-6], fluidized-bed spray coating [7-8], and emulsion/precipitation (unpublished results). Also included are common techniques such as lyophilization, jet milling [9], and spray drying [10-20].

In general, drying is required in each powder formation process but may be achieved by different driving force such as temperature or in

\*Address correspondence to this author at the PowderJect Technologies, 6511 Dumbarton Circle, Fremont, CA 94555, USA; Tel: 510-742-9700; Fax: 510-742-9720; E-mail: yuh-fun\_maa@powderject.com.

different medium such as air, precipitating/recrystallizing solvents, etc. Prior to drying, many methods use atomization to form fine droplets. Spray drying and spray freeze drying are typical examples. Either drying or droplet formation will influence on both process efficiency and protein stability. In some cases like milling, particle formation occurs after the drying process. Spray drying has been widely used for making protein inhalation powders due to its convenient, straightforward operation. Nevertheless, while the temperature and drying effects on protein stability have been extensively investigated, studies on spray-drying process optimization and its scalability were ignored. This review will also address this issue.

## II. SPRAY-DRYING

Spray drying, the process wherein a liquid feed is rapidly transformed into a dried particulate form by atomizing into a hot drying medium, is a common method for preparing solids in the chemical, food, and pharmaceutical industries in the last few decades. As the cloud of fine droplets generates a tremendous amount of air-water interfacial area, water evaporates rapidly and drying completes in a matter of a few seconds to tens of seconds depending on the scale of the dryer.

### Process Stress on Protein Stability

Studies on spray-drying of biologically active materials began with enzymes [10,12,14,21]. However, spray drying of therapeutic proteins is a more recent development [15,16,22,23]. A typical concern with spray drying of proteins/peptides is how thermally labile proteins can resist heat denaturation by hot air. This is a simple physical chemical phenomenon. During the early stage of drying where the droplet surface remains moisture saturated, i.e. 100%RH, the droplet surface temperature maintains at the wet-bulb temperature that is significantly lower than the hot air temperature. As drying continues, the droplet temperature begins to rise as water diffusion to the droplet surface cannot keep surface 100% moist. At this stage, the protein is primarily in the solid state and also the air temperature decreases significantly due to moisture uptake. Thus, thermal denaturation is not typically observed in spray-drying. However, it is a good practice to use a lower inlet air temperature to reduce the potential

thermal stress to the protein. Nevertheless, like freeze-drying, protein denaturation often occurs during drying so it is necessary to incorporate a stabilizer (e.g. sugars, amino acids) into the protein formulation [11,14]. For some proteins, spray drying can alter their secondary structure ( $\alpha$ -helix,  $\beta$ -sheet, and random coil) [24] as observed in lyophilization [25,26]. The alteration is attributed to the removal of hydration water molecules that are required to form hydrogen bonds to stabilize the protein's secondary structure [27]. Thus, in developing a biochemically stable spray-dried protein product, it is judicious to dry the protein with a substance (e.g. sucrose, trehalose) that serves as a good water-replacing agent [28,29].

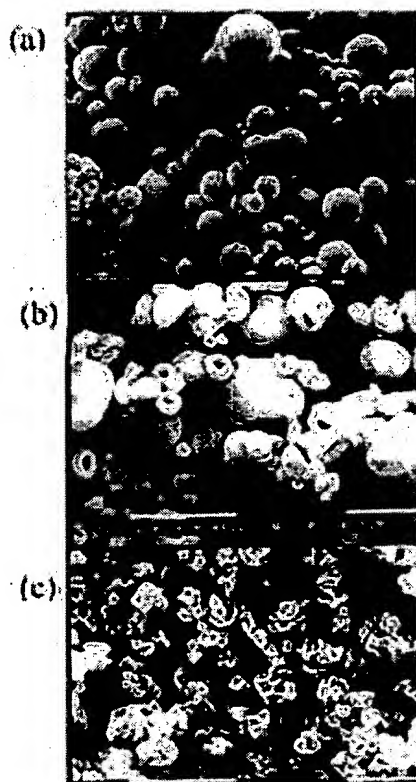
Shear stress associated with atomization and air-water interfacial stress are the two other possible stress events during spray drying. It was demonstrated previously that proteins could sustain shear rate as higher as  $10^5 \text{ s}^{-1}$  [30]. Mathematical modeling estimated that shear rate arising from atomization is at most in the range of  $10^4$ – $10^5 \text{ s}^{-1}$ , therefore, it should not be a significant factor. However, when shear stress of this magnitude is combined with air-water interface, it caused significant aggregation for air-water interface sensitive proteins such as recombinant human growth hormone (rhGH), bovine serum albumin, and lactate dehydrogenase (LDH) [19,31,32].

The structure of most proteins is more or less amphiphilic, i.e. surfactant-like structure. These protein molecules tend to be adsorbed to the air-water interface where the unusual surface energy might cause the protein molecule to unfold and to expose its hydrophobic regions; the unfolded molecules then undergo aggregation by the interaction of their hydrophobic region until precipitation [33,34]. This kind of surface denaturation has a great influence on spray-dried proteins because atomization generates fine droplets with an extremely high specific surface area ( $A$ ). It shows the relationship of  $A = 6/D_{\text{droplet}}$ , for example,  $6000 \text{ cm}^2/\text{cm}^3$  for  $10\text{-}\mu\text{m}$  droplets. A linear relationship between rhGH aggregation and  $1/D_{\text{droplet}}$  suggests that aggregation was dominated by the total air-water interfacial area [15]. Three options were found effective to minimize rhGH aggregation: addition of a surfactant to prevent insoluble aggregation, the addition of divalent zinc ions to prevent soluble aggregation, and the use of high concentrations of rhGH in the liquid feed.

### Factors Affecting Particle Shape/Morphology

Spray-drying atomizes a solution or a suspension via a nozzle, spinning wheel or disk, or other devices, through different atomizing energy such as pressure energy, kinetic energy, vibrating energy *et al.* In the case of pneumatic atomization, the size of atomized droplets increases with increasing feed rate, viscosity and surface tension of the solution and orifice size, but with decreasing air/feed ratio. Based on these facts, obtaining fine aerosol powders and system scale-up requires delicate balance, otherwise difficulties may be encountered with large-scale production. The rate of evaporation is critical to the quality of spray-dried particles. Fast drying may cause deformed or defective particles. Slow drying may result in particles too wet and too sticky. The properties of spray-dried materials also play an important role in determining the shape of the final particles. Some materials tend to form solid spherical particles while the others to form hollow, deformed (shrivelled and cenosphical) or disintegrated particles [35]. Particle shape and morphology is determined by the properties of the film/crust at

the surface of the sprayed droplets and by the drying rate. At the fast drying rate that directly correlates with the outlet air temperature, it promotes the formation of a viscous film or a dry crust at the droplet surface. This film/crust slows down the diffusion of water to the surface. As the water vapor pressure builds up, the film/crust bursts or collapses to change particle shape from its original sphericity. The properties of the film/crust include flexibility, mechanical strength, porosity, and so on. Obviously, the composition of the starting formulation, i.e. the protein and the excipients, determines the properties of the film/crust. The nature of the protein has a stronger influence on particle morphology because of its air-water interface sensitive nature. Recombinant human deoxyribonuclease (rhDNase) tends to form spherical particles with smooth morphology (Fig. 1a). Recombinant human anti-IgE antibody tends to form donut-shaped or dimpled particles (Fig. 1b). However, proteins like rhGH and bovine serum albumin (BSA) tend to form particles of raisin-like morphology (Fig. 1c). With a even pronounced influence, the low molecular weight surfactant like polysorbate-20 tends to smooth out



**Fig. (1).** SEM morphology of spray-dried recombinant human deoxyribonuclease (rhDNase) forming spherical particles with smooth morphology (Figure 1a). Spray-dried recombinant human anti-IgE antibody forming donut-shaped or dimpled particles (Figure 1b). Spray-dried rhGH and bovine serum albumin (BSA) forming particles of raisin-like morphology (Figure 1c).

the surface. The raisin-like particle surface of rhGH turned to a smooth morphology when 0.05% of polysorbate-20 was added [15]. Although the effect of particle shape/morphology on particle performance, for example, in aerosol applications remains unclear, it can play an important role when surface cohesion/adhesion of the particles dominates the dispersibility of the powder.

### Factors Affecting the Powder's Residual Moisture Content

The impact of moisture and temperature on solid-state protein stability is well documented [36,37]. In general, a protein's chemical stability decreases with increasing moisture in the solid due to changes either in dynamic activity or conformational stability of the protein, or due to water serving as a reactant and/or as a medium for mobilization of reactants [37]. As far as protein inhalation powders are concerned, their physical stability (particle size and surface morphology) is equally important as chemical stability [38]. Generally the level of moisture in the powder affects particle size and excipient crystallization significantly during long-term storage, thereby deteriorating the dispersion performance of the powder [39,40]. This might be true for all powder formulations that require strict control on particle properties. Therefore, in the development of spray-dried powder product, it is essential to understand how the spray-drying conditions affect the powder's residual moisture content and thus the powder's physical and chemical stability.

In the drying chamber, the drying force for dehydration is the difference of partial water vapor pressure (or relative humidity) between the solid surface and the environment,  $P_{\text{droplet}} - P_{\text{DA}}$ . When there is enough water initially to keep the surface saturated, the surface relative humidity is 100%. This surface vapor pressure decreases as the sub-surface can no longer supply sufficient water for surface saturation due either to slowed-down diffusion or to the reduced moisture level within the protein solid. Therefore, the final moisture level of the protein solid is determined by two factors, the nature of the material and the humidity of the drying conditions. The former is due to the interactions of water molecules with protein and/or formulation excipient molecules [41]. The number and distribution of strong and weak binding sites in protein and excipient molecules are among the intrinsic properties determining these interactions. Therefore, the moisture level in the protein solid can be primarily controlled by the humidity of the

environment where the powder is manufactured, processed, and stored, due to the equilibration between the powder's residual moisture and environmental humidity.

Under the normal drying conditions, the powders prepared by freeze drying are drier than those prepared by spray drying, 1-4% vs. 4-10% [16]. During freeze drying, the final moisture content is determined by the secondary drying step, virtually a vacuum drying process performed at near room temperature. Therefore, a long drying time is normally used to ensure that the solid is dried to its equilibrium moisture content, i.e. in equilibration with the environment. However, inside the drying chamber of the spray dryer, the droplets encounter a continuously changing environment where the drying air temperature decreases and the relative humidity increases along the chamber due to moisture uptake. Thus, the driving force for heat and mass transfer decreases and so does that for water removal. If the powder reaching equilibration with the drying air during spray drying is also assumed, the final moisture content of the powder will be determined by the relative humidity (%RH) of the air inside the collection vessel where the powder stays for the longest time. Therefore, to produce drier spray-dried powders, higher inlet (outlet) air temperatures and lower liquid feed rates are required. Many studies to produce spray-dried powders with moisture contents of less than 3% used inlet air temperatures of 140 °C or higher (or outlet air temperatures of 90 °C or higher) or the liquid feed rates of 2 mL/min or lower [24]. This represents an undesirable manufacturing condition that slows down the production rate and may impose potential adverse effects on protein denaturation [12,16]. Therefore, subjecting the powder to a secondary vacuum drying process might be a better alternative to reduce the moisture content of the spray-dried powder. Nevertheless, if this powder is exposed to a humid environment, it will pick up moisture until its moisture content equilibrates the %RH of the surrounding. Regardless of the drying method, the final moisture of the powder is determined by the environment where the powder was further processed or stored.

Another approach of improving moisture removal is to decrease  $P_{\text{DA}}$  by dehumidifying the air prior to entering the chamber since mass transfer is affected by the driving force of  $P_{\text{droplet}} - P_{\text{DA}}$ . As the relative humidity of ambient air was reduced down to 5% or lower by a dehumidifier, this additional dehumidification step does not

reduce the moisture content of the powder further [16]. However, the dehumidified drying air can improve the drying capacity, i.e. removing more water per unit time.

### Process Parameter Considerations

Many researchers studying spray-drying of proteins used bench-top spray dryers. The scale of this dryer allows a relatively small quantity of the material to be processed, which might be critical to protein research where the availability of high-valued proteins is limited. Unfortunately, the efficiency, i.e. powder collection, of this kind of dryer is low. It is particularly true for collecting powders of smaller than 5  $\mu\text{m}$ . Furthermore, a major concern with the bench-top dryer is the low product yield, normally in the range of 30-40% [12,14]. For formulation scientists, it is hard to realize that with careful design this bench-top dryer can be a useful manufacturing tool, at least for early phase clinical trials.

The most widely used powder-collection equipment is a cyclone separator, in which the particle-laden gas enters a cylindrical or conical chamber tangentially at one or more points and leaves through a central opening at the top. Solid particles, by virtue of their inertia, move toward the wall of the separator from which they are fed into a receiver. Working essentially as a settling chamber, the cyclone uses centrifugal acceleration to replace gravitational acceleration as the separating force. The centrifugal separating force can be as high as 2,500X gravity force in very small, high-resistance cyclone units [42]. Powder collection by the cyclone is governed by complex fluid dynamic behaviors in the cyclone and receiver [43]. The fluid behavior is affected by cyclone design [44]. Therefore, the study to improve powder recovery using different cyclone and receiver designs and their configurations yielded useful information in this area [20].

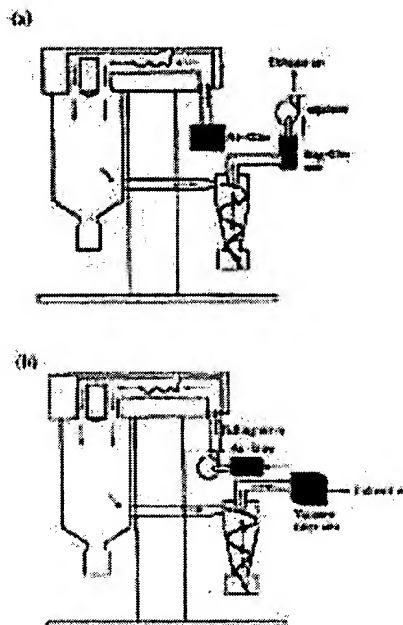
Mass balance indicated that material loss in the bench-top spray-drying system is due mostly to the attachment of sprayed droplets and dry powder to the wall of the apparatus, and the cyclone's poor efficiency in collecting fine particles. Particle adhesion to the wall mainly occurs in the drying chamber as well as in the cyclone, and is affected by the nature of the spray-dried materials and spray-drying conditions. For conditions where cyclone tends to retain a significant amount of the powder, recovering the powder from the cyclone as part of the product might be necessary if protein

stability and particle properties remain unchanged. Many researchers may find cooling down the cyclone would help maintain the protein's stability (unpublished observations from several sources). This is maybe true for very thermally labile proteins. However, despite the high temperature in the cyclone, most proteins investigated so far are stable [13,18,19]. One possible drawback with reducing the cyclone temperature is the relative humidity of the air in the cyclone will increase and result in a higher moisture content of the powder.

The design of the bench-top dryer (Büchi 190) has limitations in drying air flow, thereby limiting the batch size for spray drying. It is due to a bag-filter unit located in the down-stream of the system (Fig. 2a) through which a respirator pulls the drying air. This filter unit presents a major resistance to air-flow. Worst of all, the resistance increases during the drying process as fine particles slowly build on the bag to foul the filter. A report [20] described modifications to the dryer to improve airflow. The modifications (Fig. 2b) include removal of the bag-filter unit, relocation of the aspirator, and addition of a vacuum-filter unit. This modified system increased the capacity of drying air, which allowed droplets to be dried at a lower inlet air temperature while the outlet air temperature remained unchanged. This is important to the spray drying of heat-sensitive proteins. Both the removal of the bag-filter and the addition of a vacuum-filter resulted in almost 100% increase in the air-flow rate. This allows non-stop spray drying of 2-L batch volume (up to 60 grams of solid). Design changes such as using dual cyclones and dual receivers in different configurations and cyclones of different designs were tested and found that their effects on powder collection are minor. Also, the effect of using an anti-static treated cyclone on powder collection was found to be insignificant. All of these suggest that the bench-top spray dryer can be a useful production tool for preparing high-valued, low-volume protein products if powder collection efficiency is acceptable. Powder collection is affected more by protein formulation than by system design.

### Scale-Up Considerations

Spray drying has been used in a variety of industries to produce powders of 100  $\mu\text{m}$  or larger and has evolved into a mature technique for industry-scale production up to a few tons per day [10]. However, in the relatively young biotechnology industry, the production of high-



**Fig. (2).** The original design of the bench-top dryer (Buchi 190) (Figure 2a). The modified design of the bench-top dryer (Figure 2b) which can reduce air-flow resistance.

valued protein powders has been limited to within the laboratory. There is very little information available for considering the scale-up issues. However, the challenges for the direct application of an industry-scale spray dryer can be realized. First, any development work based on that kind of scale might be prohibitive economically because of the material costs. Also, the use of large dryers for aerosol powder ( $< 10 \mu\text{m}$ ) production raises a concern of atomization efficiency.

By definition, scale-up of a spray-drying process involves increasing the powder output while maintaining product quality comparable to the small-scale process. Several factors affect the rate of product output; these include liquid throughput, solid (protein/excipient) content in the solution/suspension formulation, and product recovery efficiency. Low recovery of aerosol particles upon spray drying by cyclone collection is a concern due to the poor deposition characteristics and the cohesive nature of the powder as a result of very small particles. Even though the general concept favors improved recovery associated with large dryers, the published results are lacking.

When liquid throughput is increased, the process to produce desired powders is limited by two factors, atomization and heating capacity. Of many atomizing mechanisms, air (two-fluid) atomization, based on kinetic energy, should be

one of the most effective methods for generating fine sprays. However, its efficiency will decrease as the liquid feed rate increases because the atomizing air will be acting on the liquid in a less homogeneous fashion. Drying capacity is measured by the rate of water removal from the dryer in the form of vapor. Drying air with more thermal energy can be achieved with higher temperatures and higher flow rates depending on the power source of the system. Larger dryers normally come with larger power supplies to boost heating capacity. However, since the outlet air temperature is the most critical parameter in the spray-drying process [17], heating capacity is limited by how high the inlet air temperature can go at a fixed outlet air temperature without compromising protein stability during the process.

A more direct way to increase powder production is to maximize the protein concentration or the total solid content in the starting liquid formulation. The solubility of the active or inactive components limits the solid content. Nevertheless, the stability of the protein at high concentrations should be monitored and studied to ensure good stability prior to spray drying. The solid concentration can also affect particle characteristics such as particle size, density, and morphology.

The dimensions of the drying chamber determines the scale of the dryer. The taller the

chamber, the longer the residence time for the droplets and larger the particle that can be produced. Under the normal conditions, the bench-top spray dryers such as Büchi-190 can only produce powders of  $<10\text{ }\mu\text{m}$ . The next scale up is the laboratory-scale spray dryer that is commercially available in different sizes. The Yamato DL-41, the Mobile Minor by Niro, and the Tower-unit by Bowen Engineering represent the commonly used models at an increasing scale, which can produce powders with a particle size of typically 15, 20, and 30-50  $\mu\text{m}$ , respectively. Dryers of larger scale are rarely used for therapeutic protein products because of limited production quantity.

### III. LYOPHILIZATION

Freeze-drying is probably the most common method for drying proteins. The lyophilization process basically consists of three steps: (1) freezing the solution; (2) primary drying to remove the majority of bulk water by sublimation removal of ice; (3) secondary drying to reduce the solid-associated moisture content of the final product to the 1-4% range by vacuum drying. The dry cake that has a volume of the original solution after a majority of mass is removed suggests a highly porous structure. Since typical lyophilization does not involve droplet formation, the dry cake can only be reduced to particles when subjected to a secondary dispersive force such as mechanical milling or grinding. Although the particle size and distribution are primarily controlled by the input energy, the properties of the lyophilized cake also play a role. These properties include the porosity, pore size and distribution, and the chemical composition of the cake.

#### Effect of Freezing Rate

Freezing is a critical step that controls the nucleation and growth of ice crystals. Nucleation of ice crystals is necessary to initiate the freezing process. In the case of homogeneous nucleation, pure bulk water freezes after being supercooled. The rate of ice crystal growth depends on the supply of water molecules from the liquid phase and on the removal of latent heat. Both are complex phenomena and can be explained with complicated thermodynamic equations [45,46]. In simple terms, with slow freezing ice nuclei have a greater chance to grow into larger crystals. On the contrary, with rapid freezing the number rather than the size of the nuclei increases. In a freezing

experiment monitored using freeze-drying microscopy, rhGH samples were frozen with a slow freezing rate ( $0.8\text{ }^{\circ}\text{C}/\text{min}$ ) and a fast freezing rate ( $10\text{ }^{\circ}\text{C}/\text{rate}$ ) rate. Their optical micrographs displayed dark lines representing the boundary of ice crystals and the freeze concentrate zones where the protein was highly concentrated. It was observed that fast freezing produced smaller ice crystals than slow freezing. Upon drying, the structure of the frozen matrix remained unchanged after drying in both cases. Furthermore, the frozen film dried at  $-30\text{ }^{\circ}\text{C}$  and 115 mTorr shows a drying front. It was observed that drying did not disturb the freeze concentrate but left the ice crystals sublimed. Therefore, in a frozen bulk the freeze concentrate would form around and between ice crystals possibly in a form of an irregular film. After drying and being subjected to a mechanical force (jet milling), the dry cake collapsed into flaky irregular-shaped particles. The particle size is reversely proportional to the freezing rate in a linear fashion, i.e. faster the freezing rate, the smaller the particle size. This holds true for several different proteins (unpublished results by Maa).

#### Effect of Protein Concentration

At higher protein concentrations, the freeze concentrate contains more protein and forms a strong porous matrix that is more difficult to reduce to small particles by mechanical forces. It has been demonstrated with both BSA and rhGH that the size of freeze-dried and milled particles increase with increasing protein concentration in a linear fashion (unpublished data by Maa). The freeze-dried cake of the same volume was denser from a higher starting concentration than from a lower concentration because the former has more mass. The dense cake is less porous and mechanically stronger; therefore, it is difficult to be dispersed into smaller particles by post-lyophilization dispersion such as homogenization or sonication.

Separate experiments investigating effects of the freezing rate and concentration on particle size indicated that the effect of freezing rate is significant only over a certain concentration range. Using BSA as an example, differences in the size of protein particles corresponding to two freezing rates, 10 and  $0.6\text{ }^{\circ}\text{C}$  per minutes, at three different protein concentrations, 1, 10 and 100 mg/ml, are presented. The particle size difference is most significant at the medium concentration, 10 mg/ml, and less significant at the low concentration (1 mg/ml). However, the trend is



reversed at the high concentration (100 mg/ml). It appears that the concentration effect outweighs the freezing rate at the high concentration. By data fitting, the mathematical relationship between particle size, protein concentration, and the freezing rate can be expressed by Eq. (1):

$$D_p = 8.88 + 0.0966 (C) (T) \quad (1)$$

where  $D_p$  is the particle size in  $\mu\text{m}$ ,  $C$  the protein concentration in mg/ml and  $T$  the freezing time in minute. This relationship holds for many proteins investigated, suggesting that This phenomenon is probably protein independent. This equation provides useful quantitative information on the particle size of the freeze-dried proteins and is particularly important for large-scale freeze drying. By controlling the freezing rate, desired particle sizes can be obtained. However, obtaining freeze-dried particles of 5  $\mu\text{m}$  is difficult. It is impractical to freeze-dry protein of very low concentrations ( $< 1$  mg/ml). The effect of controlling the freezing rate has its limit. The fastest freezing achievable is to freeze a small volume in liquid nitrogen. However, it takes about 0.5 minutes to freeze 2-m of liquid in liquid nitrogen.

#### Effect of Evaporable Excipients

Evaporable excipients can be added in the protein solution in the hope of leaving behind pores during freeze-drying to generate a microporous cake. Commonly used excipients for such a purpose are ammonium bicarbonate and alcohols. Ammonium bicarbonate decomposes to ammonia, carbon dioxide and water during drying. Alcohols sublime during lyophilization. RhGH is stable in the presence of ammonium bicarbonate and alcohol. Lyophilizing rhGH with  $\text{NH}_4\text{HCO}_3$  has been well studied. Alcohol does not appear to affect rhGH chemically or physically [17]. Also, our results indicated that rhGH remains stable when freeze-dried in ethyl alcohol with a concentration up to 10%. As far as particle size for rhGH is concerned, no significant changes in particle size were observed over the 10-200 mM  $\text{NH}_4\text{HCO}_3$  concentration range regardless of the rhGH concentration. Similarly, the presence of ethyl alcohol ranging from 1-10% did not affect the particle size. Based on the spongy and porous appearance of these excipient-containing freeze-dried cakes, it is probably that a macroporous, rather than microporous, structure is established during freeze drying.

#### Effect of the Type and Concentration of the Sugar

Sugars are commonly used to stabilize the protein during drying. To investigate if the sugar plays a role in affecting the shape and particle size of the lyophilized protein, rhGH and BSA were freeze dried with different types of the sugar. The sugars included trehalose, mannitol, and cellobiose. The results indicated that neither the type nor the concentration of the sugar affects the particle size to a great extent. In a separate experiments where trehalose of different concentrations (1-100 mg/ml) at different freezing rates was freeze dried, their effects on particle size still follow the same trend as those observed in the protein system.

To conclude, the freezing rate and the total solid concentration of the formulation can control the size of the freeze-dried protein particles. However, it produces irregular-shaped particles with a wide particle size distribution. Also, the requirement for a secondary process for particle size reduction will further decrease the efficiency of the process. The major drawback of this process is the lack of droplet formation, which can be overcome by the spray freeze drying method.

#### IV. SPRAY FREEZE DRYING

Spray-freeze drying (SFD) is a relatively new method for biopharmaceutical powder preparation. This method combines the atomization and freeze-drying processes to present a potential advantage for fine powder preparation. The principle is to atomize the protein solution into a cryogenic medium, such as liquid nitrogen, to quench the droplets. Then the frozen droplets are dried by lyophilization. This process involves no heat for drying so that heat denaturation associated with the spray drying process can be avoided. This method was used recently for aerosol applications [3].

Spray drying and spray freeze drying produced powders of different physical and aerosol dispersion properties for different protein formulations [3]. The spray freeze-dried powders had larger median particle size, larger specific surface area, and higher fine particle fraction (FPF) than the spray-dried powders. With the spray-drying condition used in this study, atomization resulted in droplets of approximately 10  $\mu\text{m}$  in median diameter. The size of these droplets shrank to 3  $\mu\text{m}$  upon water removal by hot



air during drying. Although the atomized droplets were spherical in shape, the shape of the dried particles sometimes changes depending on drying conditions and protein formulations [17]. However, in the absence of hot air drying, atomized droplets during SFD maintained their spherical shape and size upon immediate freezing, and the subsequent drying process did not affect the shape or size. Instead, the SFD process rendered particles porous. The significant increase (~40 times) in specific surface area for the SFD powder suggested its highly porous structure. SEMs of these powders (Fig. 3) confirm that spray-dried particles showed spherical but dimpled shapes (a) and spray freeze-dried particles were spherical but porous (b). The particle density of the porous spray freeze-dried powder was significantly reduced. According to the definition of aerodynamic particle diameter,  $D_a = D_s \rho_s^{0.5}$  [3], the SFD process resulted in powders of a suitable aerodynamic particle size for inhalation.

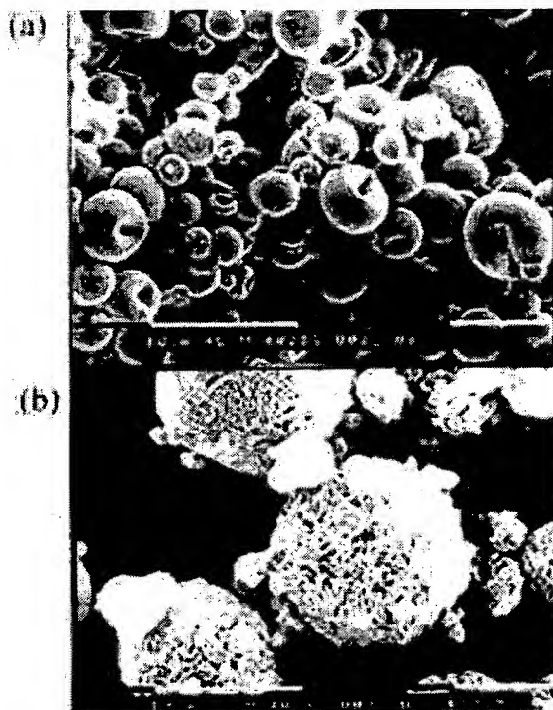
The fine particle fraction (FPF) of these four powders was commonly determined in-vitro using a multi-stage liquid impinger. Spray freeze-dried powders consistently outperformed spray-dried powders in this test. The result also indicated that the spray freeze-dried powder could deposit in the deep lung due mainly to its improved aerodynamic

properties and reduced interparticle interaction (adhesion).

Physical particle size still played a role in FPF. Between ultrasonic atomization and two-fluid atomization, the former produced a powder of the largest physical size (32  $\mu\text{m}$ ) and the smallest surface area (44.1  $\text{m}^2/\text{g}$ ). The latter resulted in smaller particles, 19  $\mu\text{m}$  (49.7  $\text{m}^2/\text{g}$ ) and 5.9  $\mu\text{m}$  (72.9  $\text{m}^2/\text{g}$ ) corresponding to atomizing air-flow rates of 600 and 1000 L/min. The FPF decreased significantly with increasing physical size. Powders with a preferred FPF (>30%) could only be produced using two-fluid atomization at an atomizing air flow rate of >1000 L/hr.

### Formulation Effect

Formulation had a strong influence on the powder's aerosol performance. Sugar alcohols with a high crystallizing tendency such as mannitol could cause particles to fuse together, thereby reducing the FPF significantly. At high concentrations of trehalose, the spray freeze-dried particle lost the characteristics of porosity. All of these typify an intimate relationship between the formulation process and formulation composition.



**Fig. (3).** SEMs of a spray-dried powder (3a) showing spherical but dimpled shapes and spray freeze-dried particles (3b) being spherical and porous morphology.

### Protein Aggregation Upon SFD

Of a few proteins spray freeze dried so far, some proteins were stable but some showed different levels of aggregation tendency, including recombinant human deoxyribonuclease (rhDNase) and recombinant human anti-IgE monoclonal antibody (rhMAB) (Table 1). Excipient-free protein powders suffered significant aggregation upon spray freeze drying, 21.6% for rhMAB and 13.6% for rhDNase. However, the same excipient-free proteins, following a normal lyophilization process (freeze dried in vials), aggregated only slightly, 4.3% for rhMAB and 1.2% for rhDNase. This suggests that the additional spray-freezing operation presented a highly stressful event to the protein. The nature of the stress may be due to events such as the shear stress, the stress of air-water interface associated with air atomization, the stress of extremely fast freezing, and so on. It has been demonstrated that shear and the shear stress associated with air atomization were low and had

little influence on rhDNase, and that rhDNase was very stable at the air-liquid interface [7,8,30,31]. The same was observed with rhMAB (data not published). Therefore, it appears that protein aggregation upon spray freeze drying occurred mainly during the fast freezing step. This is discussed in more detail below.

### Extremely Fast Freezing Upon SFD

Considering the freezing time for 1-mL liquid in a vial in liquid N<sub>2</sub> being approximately a few seconds, extremely fast freezing could be achieved by subjecting a small volume ( $5.2 \times 10^{-10}$  cm<sup>3</sup> for a droplet of 10-  $\mu$ m in diameter) into an extremely cold environment (liquid N<sub>2</sub> at -196°C). A simple mathematical model based on a steady state heat-transfer condition was derived to estimate the freezing time of a 10-  $\mu$ m droplet. The calculation suggested that the droplet froze in less than one millisecond, approximately 4 orders of magnitude

**Table 1. Soluble Protein Aggregation and Surface Area of Spray Freeze-Dried and Freeze-Dried Powders of rhDNase and rhMAB Formulations<sup>a</sup>**

Formulations	Spray freeze drying		Freeze drying
	Aggregation (%)	Surface area (m <sup>2</sup> /g)	Aggregation (%)
Excipient-free rhMAB	21.6	127.7	4.3
rhMAB:mannitol 80:20	20.8*	121.6	0.2
rhMAB:trehalose 60:40	14.6	74.6	0.3
rhMAB:trehalose 60:40 + 2% PEG	15.0	9.5	0.2
Excipient-free rhDNase	13.6	121.2	1.2
rhDNase:trehalose 80:20	6.5	110.5	0.6
rhDNase:trehalose 60:40	5.4	70.5	0.5
rhDNase:trehalose 40:60	5.7	4.4	0.4
rhDNase:mannitol 80:20	7.5*	123.7	0.4
rhDNase:mannitol 50:50	9.2*	29.9	0.9
rhDNase:mannitol 60:40 in 50mM sodium phosphate (pH 6.0)	7.2*	11.1	0.5
rhDNase:sucrose 60:40	6.3	41.5	0.5

<sup>a</sup> Spray drying and spray freeze drying conditions were described in the Method Section.

\* Mannitol crystallized as determined by X-ray powder diffraction analysis.

faster than the freezing time for 1-mL liquid in the vial. It is well known that fast freezing generates a large surface area of freeze-dried solids [48,49]. This was confirmed by SEM analysis suggesting that spray freeze-dried, excipient-free rhMAb and rhDNase powders show a very porous structure. The characteristic high porosity was also confirmed by comparing the pore size distribution and the internal surface area of the spray freeze-dried particles with their freeze-dried counterparts. Their corresponding cumulative pore volumes, 0.23 and 0.0019 cc/g, differed by 2 orders of magnitude. Their surface areas were also dramatically different, 121.2 m<sup>2</sup>/g for the SFD powder and 1.4 m<sup>2</sup>/g for the freeze-dried powder. With such a large surface area, the role of ice-water interfacial denaturation was investigated further.

### Effect of Cryoprotectants

As shown in (Table 1), compared to excipient-free formulations, all sugar excipients protected the protein against aggregation to a great extent for the freeze-dried powders and to some extent for the SFD powders. RhDNase was better protected than rhMAb. When added to the spray freeze-dried rhMAb/trehalose formulation, PEG had no additional effect. Overall, the degree of aggregation was relatively high for the spray-freeze-dried rhMAb (e.g. ~15%) despite the protection by sugar excipients, suggesting that other mechanisms than the normal freeze-drying stress events might be involved.

Based on the fact that sugar excipients lose their protective function as a result of crystallization-induced phase separation, X-ray powder diffraction analysis was used to determine sugar crystallization. All but mannitol-containing formulations remained amorphous upon SFD (formulations with \* in Table 1). Mannitol tends to crystallize upon freeze drying [50,51] when its concentration in the solid exceeds 30% (dry basis). Interestingly, mannitol in the 80:20 rhMAb:mannitol formulation was amorphous upon freeze drying but crystallized upon SFD. Another example indicating that SFD promotes mannitol crystallization was the 60:40 rhDNase:mannitol formulation containing 50-mM sodium phosphate. Sodium phosphate at this concentration could inhibit mannitol from crystallization [50,51].

(Table 1) shows protein formulations containing crystallized sugar excipients were

generally less stable compared to formulations where sugar excipients remained amorphous. Despite this, the physical state of the cryoprotectant alone cannot fully explain protein aggregation upon freezing during SFD.

### Effect of Ice-Water Interface (Surface Area)

The surface area (Table 1) varied from 4.4 m<sup>2</sup>/g to 127.7 m<sup>2</sup>/g, suggesting that formulation affected the porosity of the SFD powders significantly. However, no clear trend was observed between the surface area and protein aggregation. For example, the powder of 40:60 rhDNase:trehalose had the smallest surface area (4.4 m<sup>2</sup>/g), but its aggregation (5.7%) was comparable to that of the 60:40 rhDNase:trehalose powder that had a much higher surface area (70.5 m<sup>2</sup>/g).

SFD powders of different sizes were prepared using two-fluid and ultrasonic nozzles: 7 m (two-fluid at 1000 L/hr), 16 m (two-fluid at 600 L/hr), and 32  $\mu$ m (ultrasonic atomization). Their respective specific surface area is 74.6, 49.9, and 44.3 m<sup>2</sup>/g and their respective aggregation is 14.6, 9.3, and 0%. Apparently, the surface area does not correlate with protein aggregation mainly because ultrasonic atomization generated aggregate-free protein although this powder still had a large surface area (44.3 m<sup>2</sup>/g). This result clearly suggests that denaturation at ice-water interface played a role, but was not the only factor.

The role of ice-water interface was further examined using the rationale that adding a low molecular-weight surfactant, such as polysorbate 20, would occupy the ice-water interface and thus alleviate protein interfacial adsorption/denaturation. The formulation of rhMAb:trehalose containing 0.05% polysorbate 20 was spray freeze dried using air atomization at 1000 L/hr. The surface area of the powder remained unchanged compared to the surfactant-free powder. Even though the aggregate content decreased, the level of aggregation was still high (8.7%), again suggesting that denaturation at ice-water interface was not the only factor affecting protein stability.

Based on the results above and a freeze/thaw study, a freezing-related stress event, arising possibly from a shear stress associated with phase separation between ice and freeze concentrate, might be most responsible for protein denaturation upon SFD. This freezing stress generated at the freezing front (ice-water interface) has an adverse effect on the two model proteins only at a freezing

rate beyond a critical point, in the range of  $8.4 \times 10^{-4}$  and  $3.4 \times 10^{-3}$  sec (for droplet diameter between 16 and 32  $\mu\text{m}$ ). When the ice front advances faster than this critical rate, it might alter protein conformation that leads to aggregation upon drying. However, it appears that these conformational changes are reversible upon relaxation of the stress by thawing. To test this hypothesized mechanism, we performed a series of experiments to anneal the spray-frozen powder of 60:40 protein:trehalose at temperatures (-5, -10, and -15°C) higher than the primary drying temperature (-25°C) for one hour before the lyophilization cycle began. This annealing process decreased the powder's surface area and protein aggregation. The higher the annealing temperature, the smaller the surface area and the lower the aggregate content. Annealing at temperatures near the melting point caused large ice crystals to grow at the expense of more energetically unfavorable small crystals (a migratory recrystallization process), thereby reducing the surface area of the particle. Since surface area played only a limited role, the reduction in aggregation by annealing could be attributed to the relaxation of the proposed stress.

Another advantage of the spray freeze-drying method is the potential for producing powders of sub-micron size. After freeze drying, the size of these porous spheres that are physically weaker than that of the solid spheres, could be further reduced using a secondary process such as milling or homogenization.

## V. PULVERIZATION

Mechanical pulverization for particle production has been widely used in the mineral, ceramics, and chemical industries. In a broadest definition, pulverization, or comminution, is the process of using a variety of mechanical mechanisms for reducing the size of particles or aggregates. These may include the mechanism of cutting, chopping, crushing, grinding, milling, micronizing, or their combinations, and they depend primarily on the type of equipment used such as roller mills, hammer mills, cutter mills, ball mills and fluid energy (jet) mills [1]. Milling produces particles normally in the range of 10-50  $\mu\text{m}$ . To obtain finer powders, two factors must be recognized beforehand; energy consumption and material properties. The fineness to which a material is ground has a marked effect on its production rate, cost and required energy. Material properties, such as hardness and brittleness, affect

grindability significantly. The Moh's scale measures material's hardness from 1-10. Materials scaled from 1 to 3 are considered as soft, 4-7 as intermediate and 8-10 as hard. The size of hard and brittle materials can be effectively reduced by the action of rolling and impact while the action of shearing and tearing is more effective for soft substances. A vast majority of pharmaceutical powders falls in the categories of soft and intermediate hardness. In view of the fact that most proteins are soft substances, in principle, equipment capable of delivery attrition action in addition to impact should be employed.

It was estimated that particle size reduction by pulverization has an inherent limitation due to two factors; plastic deformation and the difficulty of stressing fine particles to their breaking point [52]. The latter requires that the particle have enough stored stress energy to allow a crack to propagate. The former is considered particularly important in limiting the particle size during comminution because, to some extent, viscoelastic properties exist in all substances, even in fine glass spheres. During particle size reduction operation, the applied stresses are consumed in the form of surface energy associated with newly created particle surfaces, internal free energy associated with lattice changes and heat. Overcoming the first two energy barriers results in fracture, i.e. particle size reduction. Most of the energy expressed as heat is consumed in the viscoelastic deformation of particles, friction, and in imparting kinetic energy to particles. As the particle size decreases, more energy is converted into heat and gradually dominates the energy distribution. It was reported that small particles behave more plastically than larger particles; therefore, small particles store more compressive energy and crushing energy per unit volume [52]. This information suggests that it becomes increasingly difficult to reduce the size of small particle at the same energy input. As to how much heat may be generated during pulverization, it was analyzed that the temperature rises at a propagating tip of a breaking particle. It was estimated that irreversible deformation occurs in a zone of radius about 30 Å running along at the cracking tip where the energy released later could increase temperature as high as 1500°K [53]. This data proved that plastic deformation can limit the grinding size attainable and suggested that methods other than pulverization must be used if particles finer than 1  $\mu\text{m}$  are desired. In addition, the heat generated during pulverization raises concerns for heat sensitive materials such as proteins.

The jet pulverizer (fluid-energy mill) is one of the most powerful pulverizing methods and is capable of comminuting particles to a size  $< 10 \mu\text{m}$ . Mechanically the jet mill involves no moving parts. The size reducing force of the system is associated with the kinetic energy of high-velocity elastic fluids, such as air or an inert gas. The starting material is accelerated to relatively high speeds when swept into violent turbulence by the sonic or ultrasonic velocity of the streams. Particles circling in the reduction chamber return to the impact zone to have head-on collision with feed particles. The impact due to high speed collision causes fracture of the particles. Particle classification is either determined by the centrifugal force where particles smaller (lighter) than a specific size (weight) to be carried away from the reduction chamber by the stream. The heavier or larger particles will return to the impact point for further pulverization.

The effectiveness and minimal contamination make jet milling choice of size reduction techniques for pharmaceutical powders [54]. Furthermore, the jet mill comes of different sizes, which allows the production of small batches of expensive proteins [9]. However, using pulverization for protein powder production remains an unexplored area. There is very little published information available until recently [9]. It identified five milling parameters affecting the particle size, including inlet fluid pressure, powder feed rate, bulk powder morphology, material hardness, starting size distribution with the milling pressure being the dominating factor. This study also suggested that a wide range of undisclosed peptides and proteins with molecular weights in excess of 25 kDa are amenable to milling. However, exceptions exist. Lyophilized rhGH and rhDNase suffered aggregation upon milling (unpublished results by Maa). Lowering the milling temperatures using pre-chilled nitrogen in an attempt to minimize aggregate formation was not successful. The long-term (up to 6 months) stability of the milled rhDNase powder was measured by storing the powders at 5 and 37°C. Powders stored at 37°C aggregated faster with increasing milling pressures while the milling pressure had less influence on powders stored at 5°C.

Pulverization remains unexplored for protein powder preparation. It can be effective for reducing particle size to be smaller than 5  $\mu\text{m}$ . However, its control (efficiency) on producing powders of larger sizes and different morphology is questionable. Intuitively, the process effect on

protein stability is relatively mild but localized hot spots during particle cracking may cause protein denaturation.

## VI. SUPERCRITICAL FLUID ANTI-SOLVENT

Thermodynamically, the vapor-pressure curve in a 3-phase diagram indicates that an increased pressure is needed to liquefy a gas when its temperature is increased. At high pressures the gaseous phase becomes more dense, and eventually it is impossible to distinguish between the gaseous and liquid phases. Under such a condition the temperature and pressure of the system reach the critical point ( $T_c$ ,  $P_c$ ). The critical temperature is the highest temperature where a liquid phase may co-exist in equilibrium with a separate vapor phase. Also, the critical point is the climax of the region where the liquid and vapor phases co-exist. Supercritical fluids (SF's) are materials whose temperature and pressure are higher than the corresponding critical point values ( $T_c$ ,  $P_c$ ). Supercritical states are specified by  $T_r$  and  $P_r$  where  $T_r = T/T_c$  and  $P_r = P/P_c$  and normally  $1.01 < T_r < 1.10$  and  $1.01 < P_r < 1.50$  [55]. SF's have special thermophysical properties, ranging between the gas and liquid extremes. For example, SF's have liquid-like densities but very large compressibility, higher thermal diffusivities and viscosities than liquids.

Currently, SF's have been applied in destruction of hazardous wastes [56], extraction of organic contaminants from soils [57], enzymatic catalysis in nonaqueous media [58], particle formation by rapid expansion [59,60]. All these processes depend on the solvent power of SF's. Yeo *et al.* [5] used the concept of supercritical fluid antisolvent expansion to prepare insulin powders. The solution of insulin in dimethylsulfoxide (DMSO) was sprayed through a nozzle into a supercritical  $\text{CO}_2$ -filled precipitator. When contacted with the supercritical  $\text{CO}_2$ , small liquid droplets were expanded and DMSO was dissolved in the SF which, however, served as an antisolvent to insulin. After the evaporation of DMSO into the supercritical  $\text{CO}_2$ , the insulin powder precipitated. This method could produce spherical particles of 1-3  $\mu\text{m}$ . The system could be operated continuously and semibatchwise. In continuous operation, no significant influence of temperature and concentration on the particle size was observed, and the nature of the solvent had no effect on the morphology of the resulting powders.

In the semibatch process, a slow CO<sub>2</sub> injection rate, i.e. the slow solution expansion, favored the growth of larger particles. This suggested that the rate of expansion could be used to control particle size. The biological activity of the precipitated insulin was determined by an animal test on rats. The blood glucose levels in rats were measured after the injection of the insulin solution. It was found the activity of the processed insulin is indistinguishable from that of the starting material, irrespective of processing conditions.

Though successful, the SF antisolvent method has its disadvantages. The system is complicated, involves complex equipment, and requires elaborative controls. Also, only a limited number of proteins is soluble in aprotic solvents such as DMSO and dimethyl formamide (DMFA). Recently, the same group of researchers produced catalase and insulin microparticulate powders by this method using 90% ethanol and 10% water protein solutions and supercritical CO<sub>2</sub> [61]. Therefore, this method can be applied to more proteins if appropriate solvents and processing conditions are identified.

To overcome the problem of protein solubility and stability in organic solvents, the SEDS (solution enhanced dispersion by supercritical fluids) method was developed. It has a major advantage in that by changing the processing conditions directed changes in particles properties, including particle size can be achieved [62]. In a single step operation, a drug solution is processed into a micron sized particulate product with narrow size distribution that is uncharged, solvent-free, crystalline [63]. There are manufacturing advantages including a totally enclosed system, reduced solvent requirements compared with conventional crystallization, and a high degree of consistency between batches.

It appears that the SF relevant methods have been proven to be effective for producing fine powder for aerosol applications. However, their capability in controlling the particle size requires further demonstration. Furthermore, the database concerning the process effects on protein stability needs to be built. From the formulation perspective, its feasibility in systems involving multiple components remains unclear.

## VII. PRECIPITATION

Precipitation is one of the oldest methods for recovering and purifying proteins. It is still

commonly used today. In a broadest term, precipitation is the reduction of solute solubility by the addition of any extraneous components or the changes of processing conditions so that most of the solute molecules can no longer stay in the solution. In this sense, SF antisolvent, like a variety of other laboratory-scale methods, is also a precipitation method. Protein solubility is determined by the balance between the ionizable groups and the hydrophobic components on the surface of the protein. The following five precipitation methods have been developed based on adjusting the system temperature, ionic strength, pH, dielectric constant on large scales: salting-out, precipitation by polyelectrolytes and nonionic polymers, isoelectric precipitation, and precipitation by organic solvents [64].

### A. Salting-out

This involves the addition of neutral salts to the protein solution. High concentrations of salt are used to affect the balance between the electrostatic forces tending to keep the protein in solution and the hydrophobic forces that cause the protein to agglomerate and precipitate in an aqueous environment. Salt ions tend to remove the water that effectively shield the hydrophobic areas, allowing protein-protein interactions. In fact, most of the proteins exhibit an initial salt-in region at low ionic strength, and then experience the salt-out effect as the ionic strength increases [65]. This phenomenon can be explained quantitatively by:

$$\log(S/S_o) = 0.509|z+z-|*I - K'I$$

where *S* and *S<sub>o</sub>* are the apparent and thermodynamic solubilities, *z<sup>+</sup>* and *z<sup>-</sup>* the ionic charges of the cation and anion, *I* is the ionic strength and *K'* is a positive constant dependent on the nature of the solute and the electrolyte.

### B. Precipitation by Nonionic Polymers and Polyelectrolytes

Nonionic polymers and polyelectrolytes cause protein precipitation through a combined mechanism, volume-exclusion and the reduction of the water of solvation. High-molecular-weight polymers of polyethylene glycol have been widely used for protein precipitation on both the laboratory scale and pilot plant scale. Polyelectrolytes, such as polyacrylic acids, polysaccharides and polyphosphates, have been used for small-scale protein purification. So far

this method is mainly for purification purpose and has not been used for the preparation of protein powders.

### C. Isoelectric Precipitation

This is based on the fact that proteins exhibit reduced solubility at their isoelectric point. The isoelectric point is the pH at which the molecule has a net zero charge; therefore, the hydrophobic forces dominate the electrostatic forces, enhance protein-protein interactions, and cause protein agglomeration and precipitation. This is not considered a strong precipitation technique due to two factors: (1) the limited pH range at which proteins are stable may be difficult to control; (2) the solubility of many proteins is not negligible even at PI, which may result in significant loss.

Using rhGH as an example, the protein precipitated from a 0.1-M acetate buffer by adjusting its pH from 8.2 to 5.2 (rhGH's PI). After the precipitate was collected and lyophilized, the size of the solid rhGH was determined to be 23.2  $\mu\text{m}$ . The processed rhGH maintained its stability (the monomer content was >90% depending on the formulations).

Although the methods above are useful for obtaining solid protein products, their control on particle size and size distribution is difficult. No systematic studies have been reported so far. The method below proposed by the authors might be effective for producing powders with a controlled size, particularly fine powders.

### D. Emulsification and Precipitation

Particle size reduction in the liquid state requires less energy than that in the dry state (e.g. pulverization). The generation of liquid droplets by atomization has been described earlier. Liquid-liquid emulsification is another approach, which has been the basis of multi-phase microsphere preparation for sustained-release applications [66,67]. Two immiscible liquids are mixed with energy input to form fine droplets (the dispersed phase) in the dispersion phase. The droplets turn into hardened or dry particles after a drying process such as solvent extraction, evaporation, precipitation, etc. The particle/microsphere size is controlled by the type and magnitude of the input energy [68,69,70]. Recently, various types of liquid-liquid emulsification such as static mixer

[71], rotor/stator homogenization [72], sonication, and microfluidization [73] were evaluated.

For macromolecules having limited solubility in an organic (nonsolvent), the addition of aqueous solute solution decreases the dielectric constant of the solution, so the system becomes less polar in character. This reduces the solvating power of the water surrounding the solute, enhances solute-solute interactions, and results in solute precipitation. To be effective, this nonsolvent must be miscible with water and hydrophilic in nature to avoid denaturing the solute, protein in particular. In a solvent/nonsolvent system, the initial solute concentration, the type and the volume ratio between solvent and non-solvent, the temperature are the most important parameters. However, this simple precipitation method is also difficult to control the particle size and results in irregular-shaped particles. Using atomization to produce droplets prior to precipitation resulted in larger particles with a broad particle size distribution, suggesting that forming stable droplets before and during precipitation is critical.

To improve droplet stability, the protein solution can be emulsified, for example, by homogenization in a dispersion phase. Sometimes, an emulsifier is needed to stabilize the emulsion. After emulsion formation, it is diluted in another nonsolvent for particle hardening or formation. Using BSA as an example, the aqueous BSA solution was emulsified in an organic solvent such as ethyl acetate or methylene chloride containing a polymeric emulsifier such as poly(methyl methacrylate) (PMMA) or poly(D,L-lactic-co-glycolic acid) (PLGA). The emulsion was then diluted in another nonsolvent such as acetone and tetrahydrofuran (THF). Acetone and THF, miscible with ethyl acetate/methylene chloride and water and being able to dissolve the polymeric emulsifier, are the nonsolvents to BSA.

This method has been demonstrated to be effective in producing fine protein powders. However, more efforts are needed to evaluate protein stability in this system. Normally active protein molecules are folded, with their polar residues pointing outside and the hydrophobic residues buried inside. As the dielectric constant is reduced, the protein responds by unfolding to an inactive form that places the hydrophobic residues in an external position. Therefore, the addition of organic solvents could decrease protein solubility and irreversibly denature proteins. Solvents that have the good balance between its effect on protein stability and an adequate hydrophilic



character are the better candidates for protein precipitation. The most commonly used organic solvents are alcohols, such as methanol, ethanol, isopropanol etc., and acetone. The effect of alcohols and glycols on protein denaturation has been studied [74], and the interaction of alcohols with proteins was also investigated [75]. Barritault et al used acetone precipitation to isolate of ribosomal proteins [76]. The properties of proteins in nonaqueous solvents, such as strongly and weakly protic solvents, were extensively reviewed [77] who concluded that native conformations of the proteins are always altered in nonaqueous solvents, and the conformation of the protein may be different in different solvents. Therefore, it is impossible to predict the behavior of proteins precipitated from different solvents, not to mention different proteins precipitated from a same solvent. Another potential limitation to this method is the solubility of the material to be precipitated. Solubility too high may either fail the whole process or result in products of a low yield. For systems involving more than one component, normally the case with protein formulations, the product of a different composition might be yielded for each excipient has its own solubility in the organic solvent.

## VIII. FLUIDIZED-BED SPRAY COATING

All methods described above involve droplet or particle formation, which plays a critical role in powder characteristics. For applications requiring specific particle properties, coating a protein formulation to a (seed) powder of well-defined particle characteristics might be an innovative and effective approach.

For particle coating, batch-type fluid-bed processors have long been used in the pharmaceutical industry for preparing solid dosage forms of non-protein-related drugs [78,79]. Due to recent advances in the development of fluid-bed coating systems such as the Würster spray coater, it allows powders as small in size as 50  $\mu\text{m}$  to be coated and has further expanded the application of powder coating in the pharmaceutical industry [80,81,82]. Even so, there is very little information in the literature regarding the process of coating therapeutic proteins onto substrate microcarriers. The publication of this nature first was reported by Maa *et al.* [7,8]. The feasibility of coating rhDNase onto lactose microcarriers as small as 50  $\mu\text{m}$  using a laboratory apparatus was investigated

based on four criteria: low particle agglomeration, high product yield, strong coating integrity, and high protein stability.

A coating layer of increasing thickness was observed under SEM as coating time increased. The protein content in the coating was confirmed spectrophotometrically. Coating integrity (mechanical strength) was examined under a shaking and an aerosolization condition. The coating remained intact after such treatment. During coating some particles agglomerated to form large particles. Agglomeration occurred because adhesion force between particles overcame the separation force by the inertia of particle movement in the chamber. Agglomerated particles usually lost their original powder properties. Therefore, low particle agglomeration is one of the important criteria for determining the quality of the product. It is well known that the larger the particle the less the agglomeration. Also, particle flow in the chamber normally improves with the scale of the coater. Therefore, technical challenges exist for coating a high-valued protein material onto small particles in a small batch size [8].

The excipient-free protein (rhDNase) after coating showed decreased bioactivity and increased aggregation (noncovalently bound). It was found that such denaturation occurred primarily due to exposure to high temperature. As a speculative mechanism, the inner surface of each coating layer might experience a higher temperature than the outside surface. It was further demonstrated that adding calcium ions to the protein solution could minimize this thermal denaturation. Scanning microcalorimetry suggested that calcium ions escalates the thermal denaturation temperature of rhDNase by 10  $^{\circ}\text{C}$ . Other stabilizers might be effective although not investigated. In general, compared to spray drying, spray coating renders the protein equivalent stress except a higher degree of thermal stress.

The current commercial fluid-bed spray coaters can process powders of 0.5-100-kg batch. Generally, the performance of the coater increases with coater scale. For coating of small scale, high-valued protein products, bench-top spray coaters are available for processing powders of smaller than 500-gram batch. However, its performance is limited for particles of <100  $\mu\text{m}$ . The high-performance bench-top coaters are currently been developing by many manufacturers.

**Table 2. Advantages and Disadvantages of Different Methods for Powder Preparation**

Method	Advantage	Disadvantage
Freeze-drying	High yield Convenient operation Aseptic process Expensive process	Poor particle size control Irregular-shaped Broad size distribution
Spray-drying	Good particle size control Spherical-shaped Easy and convenient operation	Yield dependent on formulation Heat inactivation Surface denaturation
Spray-freeze drying	Good particle size control Spherical shape High yield Good aerosol properties	Poor density control
Supercritical fluid	Good particle size control Spherical shape	Complex process Limited solubility in organic solvents
Pulverization	Easy operation High yield	Poor particle size control Irregular-shaped Broad size distribution
Precipitation	Simple & convenient operation High yield	Poor particle size control Irregular-shaped Broad size distribution Protein denaturation Difficult to handle multi-components
Emul./Precip.	Easy to control Particle size, < 3 $\mu$ m Spherical shape Applicable to a variety of materials	Yield limited by solubility Protein denaturation by org. solvent Difficult to handle multi-components

Emul./Precip.: Emulsification followed by precipitation

## IX. COMPARISON OF ALL THE METHODS

The suitability and selection of a powder formation process relies on the need for specific applications. There are a few criteria for evaluation: control on particle size and size distribution, process efficiency (yield), powder flowability, scalability, long-term powder physical stability, and long-term protein biochemical stability. Based on this, (Table 2) summarizes the advantages and disadvantages of the individual method. For convenience and simplicity, lyophilization and spray drying are the methods of choice. To obtain fine (< 5  $\mu$ m), spherical particles, the spray drying and emulsification/precipitation methods outperform other methods. Please note that protein formulation and the powder method affect each other and should be considered on a case-by-case basis. No single method is suitable for all proteins.

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